LOCALIZATION OF NUCLEOCAPSID (NP) ANTIGENIC SITES BY USING A PANEL OF MONOCLONAL ANTIBODIES AGAINST THE RECOMBINANT NP OF NEWCASTLE DISEASE VIRUS

R. AHMAD-RAUS[1*], A.M. ALI [2], W.S. TAN[3], M.S. HAMZAH[1], M. ESHAGI[3] AND K. YUSOFF[3]

[1]Faculty of Engineering, International Islamic University of Malaysia, Jalan Gombak, 53100 Kuala Lumpur. [*]e-mail: rahaar@iiu.edu.my Fax: +6-03-61964442

[2]Faculty of Agriculture and Biotechnology, Universiti Darul Iman Malaysia, Gong Badak, 21300 Kuala Terengganu, Terengganu

[3]Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 Serdang, Selangor

ABSTRACT **:** Three different NP antigenic sites were identified using deleted truncated NP mutants purified from *Escherichia coli*. One of the antigenic sites was located within amino acids 441 to 489 of C-terminal. Two other antigenic sites located within the N-terminal of NP protein from amino acids 26 to 121 and 122 to 375 residues. Identification of NP antigenic sites not only elucidates the NP sequences that are responsible in eliciting immune response, indirectly it also revealed which sequences are exposed on the NP herringbone-like structure.

Keywords: *Monoclonal antibody; nucleocapsid; antigenic sites; Newcastle disease virus*

1. INTRODUCTION

Nucleocapsid (NP) protein is the major component of Newcastle disease virus (NDV). Under electron microscopy, it resembles herringbone morphology with spikes protruding from a central channel which is in common with other members of *Paramyxoviridae* familiy. Trypsin digestion of NP does not disrupt the structure of the NP, but does release a 12 K molecular weight fragment [1].

It plays a critical role in the replication of the virus in the host cells. Together with the viral genome, it forms a NP-genome complex which acts as a template for genome synthesis and transcription. This complex binds to phosphoprotein-L protein (P-L) complex which acts as a polymerase to synthesize RNA genome [2]. The switch between replication and transcription by these complexes however are still not fully solved yet. During virus assembly, the NP is most likely to interact with the M protein [3]. Binding of the M protein to NP protein would mediate the interaction between the NP and the viral lipid membrane which results in the budding and release of virus progenies from the cell plasma membrane [4,5].

NP is composed of 489 amino acids with a predicted molecular weight of 53 kDa and is encoded by a 1470 bp NP gene [6]. Based on the amino acid sequence analysis, the NP of NDV shares a high degree of homology (91-98%) with those of the other strains of NDV. The N-terminal region of the NP up to residue 401 is highly conserved among NDV strains and variability among the NPs has been detected within the C-terminal portion similar to what has been reported for other members of the *Paramyxovirinae* [7]. However, recent study has shown that two regions from residues 444 to 459 and the final 8 amino acids of C-terminal of NP are conserved [8]. The NP of NDV was shown to be moderately homologous (30-40%) among the other members of the *Rubulavirus* [6]. In comparison with the other two genera within the P*aramyxovirinae* subfamily, *Paramyxovirus* and *Morbillivirus*, the NP protein of NDV shares 27% homology and the length varies from 489 to 551 amino acid residues with the predicted molecular weight of 53-56 kDa. The central segment of the NP protein among 27 strains in the *Paramyxoviridae* remained conserved compared to the other regions of NP protein [6].

The NP was successfully self-assembled to form herringbone-like particles that closely resembled those isolated from intact virions when expressed in baculovirus [9] and *E. coli* [10]. Similar observations were reported for the NP protein of Sendai and measles viruses [11,12]. However, apart from herringbone-like particles, ring-like particles were also observed in the expression of NP in *E. coli* [10]. Western blot analysis showed that the expressed NP was indistinguishable immunologically from virion-derived NP [13].

To date, mAbs against NP of NDV have been produced in several laboratories around the world [14, 15, 16, 17]. Most of them used the antibodies for detecting antigenic diversity and similarities among NDV strains [18, 16, 19]. One study however, managed to delineate the antigenic sites recognized by the antibodies [20]. Unfortunately the exact locations of the antigenic sites were not determined. Therefore the present study was carried out to identify its antigenic sites by using Western blotting.

2. MATERIALS AND METHODS

2.1 Full length and truncated NP construct

Full length and four truncated NP genes used were as described in Kho *et al.* (2001a) [6] and Kho *et al*. (2003) [21]. The coding sequences were cloned in pTrcHis2 (Invitrogen, USA) and transformed into *Escherichia coli* strain TOP10 (Invitrogen, USA). The expression of the genes produced full length NP, two C-terminally truncated NP (NP_{1-440} and NP_{1-375}) and two N-terminally truncated NP (NP_{26-489} and $NP_{122-489}$) mutants as shown in Figure 1.

2.2 Purification of full length and truncated NP proteins

The purification of the full length and deleted NP mutants from *E. coli* were described previously by Kho *et al*. (2001b) [10] and were performed with slight modifications. Bacterial cultures 250 ml were grown at 37^{o}C in LB broth until the culture reached A_{600} around 0.6 to 0.8. After induction with 1 mM IPTG for 5 h, the cells were harvested by centrifugation at 10,000 x*g* for 10 min at 4^{o}C. The pellets were lysed in lysis buffer [50mM Tris (pH 7.9), 0.1% Triton X-100, 0.2 mg/ml lysozyme, 4 mM $MgCl_2$], treated with 5 µg/ml RNase and 5 µg/ml RNase DNase (Amresco, USA) for 30 min at room temperature and followed by sonication (30 s) at high frequency for five times. The cell extracts were recovered by centrifugation at 20,000 x*g* for 20 min at 4^{o}C and precipitated by ammonium

sulphate (0 to 60% saturation). The precipitates were pelleted by centrifugation under the same conditions and dialyzed extensively in dialysis buffer (50 mM Tris, 100 mM NaCl, pH 7.8). The dialyzed solutions were carefully layered to 10-50% sucrose gradient and centrifuged at 110,000 xg for 5 h at 4°C. Fractions containing the full length NP were pooled, dialyzed and then concentrated with a 100 kDa cut-off Centricon centrifugal filter (Milipore, USA) for immunization purpose. While fractions containing NP deleted mutants were kept at 4°C.

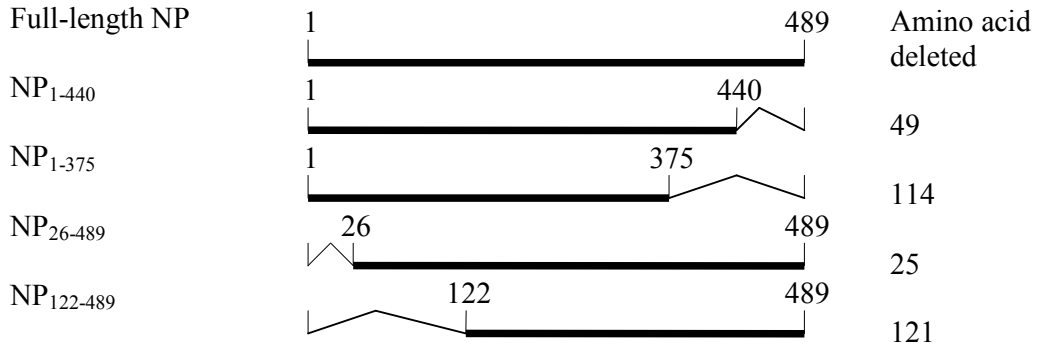

Figure 1: Schematic representation of the deletion mutants of NP. Thick lines represent the protein product of each truncated NP gene and the amino acid positions are indicated on top of theses lines. Angled lines indicate deleted regions.

2.3 SDS-PAGE and Western Blot

Purified NDV or NP proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) [21]. After the electrophoresis, the fractionated proteins were blotted onto a nitrocellulose membrane (Osmonics, USA) and Western blotting was performed according to Harlow and Lane (1988) [23] with some modifications. The blotted membranes were incubated in culture fluids of mAbs instead of diluted serum before incubation with secondary antibody conjugated to alkaline phosphatase. Nitro-blue tetrazolium chloride (NBT; Amresco, USA) and bromochloroindolyl phosphate (BCIP; Amresco, USA) were used as a substrate solution. Polyclonal antiserum against NDV or NP was used as a positive control.

2.4 Software Analysis

Amino acid sequence of NP of NDV strain AF2240 was analyzed using the antigenic index (Kolaskar and Tongaonkar method) [24] and hydrophobicity plot (Kyte-Doolittles method) [25] for prediction of antigenic sites. Higher peak in antigenic index means higher probability of the sequence to be antigenic. For hydrophobicity plot, peak with positive value indicates hydrophobic sequence and the negative value, hydrophilic.

3. RESULTS AND DISCUSSION

3.1 Full length and truncated NP proteins

In this method, two C-terminally truncated NP (NP_{1-440} and NP_{1-375}) and two N-terminally truncated NP (NP_{26-489} and $NP_{122-489}$) mutants were used (Figure 1). The

recombinants of full length and truncated NP constructs produced by Kho *et al.* [6, 28] were checked for their truncated NP genes by PCR before the respected proteins were purified. Both the full length and deleted NP mutants were purified using ammonium sulphate precipitation and sucrose gradient ultracentrifugation and the purified full length and NP mutants obtained were similar as Kho *et al.* [28] (data not shown).

3.2 NP antigenic sites

For localization of NP antigenic sites, purified deleted NP mutants were used. The purified full length and truncated NP proteins were analysed with SDS-PAGE, transferred to a nitrocellulose membrane and probed with all of the mAbs culture fluids and polyclonal NP mouse serum separately. The polyclonal antibody which served as a positive control, bound to all linearized NP mutants indicating that some of the epitopes in all of the mutants were not conformational (Figure 3a). MAbs a2, a2s and b2 recognized only those NP mutants containing only the C-terminal end (Figure 3b). Deletion of 49 amino acid residues from the C-terminal end of NP totally abolished the binding of these antibodies with the mutants (NP_{1-440}) indicating that the NP antigenic sites are located between, amino acids 441 to 489. MAbs b3 and b4s reacted with all of the C- truncated mutants but not with all of the N-truncated mutants suggesting that the antigenic sites were located in the N-terminal portion (Figure 3b). Deletion of 25 amino acids (NP_{26-489}) from the N-terminal end did not affect the binding of both antibodies, however further deletion of 121 amino acids ($NP_{122-489}$) abolished the binding. This shows that the antigenic sites recognized by mAbs b3 and b4s are located within amino acids 26 to 121. On the other hand, mAb c1 bound to all of the C- and N-truncated mutants suggesting that all the deleted amino acid sequences are not part of the epitope for this antibody (Figure 3c). This indicates that antigenic sites may be located from amino acids 122 to 375.

In previous study, Panshin *et al*. (2000) [25] has delineated at least three antigenic sites on the NP using nine NP mAbs by a competitive binding assay. However, the exact locations of these have yet to be mapped. In this study, by using deleted NP mutants purified from *E. coli* to locate the NP antigenic sites, three different antigenic sites were recognized by six mAbs, one at the C-terminal of NP and two at the N-terminal of NP.

The antigenic site at the C-terminus of NP which was recognized by mAbs a2, a2s and b2 was localized within amino acids 441 to 489. Based on the amino acid sequence analysis of NP of NDV, this antigenic site was located within the highly variable region of NP [7]. In contrast, cross-reactivity analysis showed that the epitopes of these mAbs appear to be within the conserved sequence of NP. It seems that the antigenic site determined by the Western blot analysis was not in parallel with the cross-reactivity analysis. Nevertheless, recent amino acid sequence analysis on Japanese isolates revealed that there were two smaller regions within the NP variable region (444 to 459 residues and the final 8 amino acids of the C-terminal) are well conserved among the strains [8]. Perhaps, the epitopes recognized by these mAbs may be localized within these two conserved sequences instead of the variable sequences within the amino acids 441 to 489 region. Interestingly, antigenic software analysis showed that amino acids 446-452 which overlapped with one of this NP conserved region is antigenic (Figure 4, *3*). Although the region seemed to be quite hydrophobic (Figure 5), the B-cell immunodominant epitope of NP of NDV was apparently also located on this site from 447 to 455 [29]. It is therefore possible that the exact epitopes of these three mAbs might be located within residues 446 to 455. However, further studies using synthetic peptides covering this region are required to verify the locations.

Unlike the antigenic NP sites of Sendai virus which are restricted to the C-terminal of NP protein [26], our study shows that the antigenic regions of NDV NP are also located at the N-terminal of the NP protein. Two non-overlapping antigenic sites of NDV NP were located from residues 26 to 121 (identified by mAbs b3 and b4s) and from residues 122 to 375 (bound by mAbs c1) of the N-terminal of NP protein. . Interestingly, these two antigenic sites determined by the Western blotting were overlapped with the amino acid sequences which are predicted to be the most probable epitopes (amino acids 68 to 81 and 199 to 234; Figure 4, *1* and *2*) by antigenic software analysis. However, further investigations to narrow down these two antigenic sites are required in the future so that a better understanding of antigenic structures of NDV NP could be obtained.

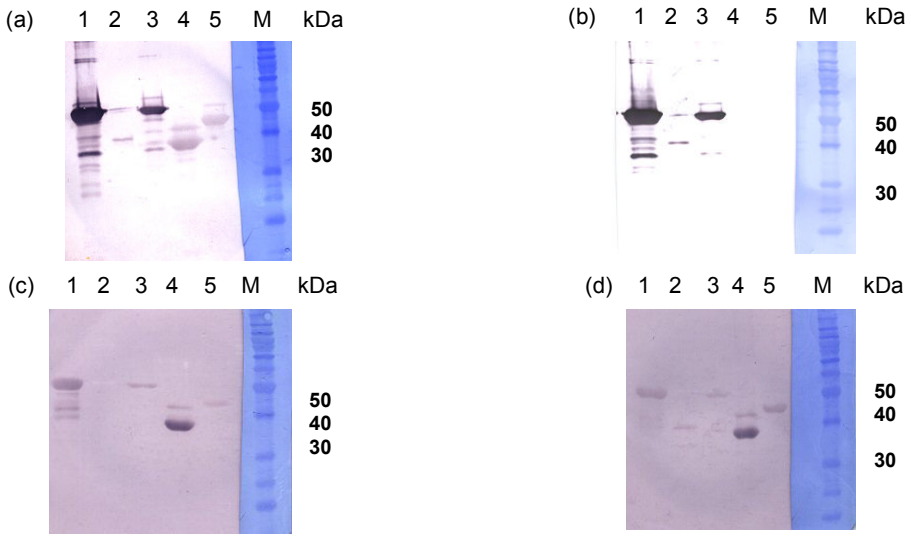

Figure 3: Western blot analysis of the truncated NP.
Purified full-length of NP and truncated NP mutants were fractionated on SDS-PAGE, electrotransferred to nitrocellulose membrane and probed with polyclonal NP serum and each of six mAbs. Lane 1 represents full-length NP and lanes 2 to 5 represent both N- and C-terminally truncated $NP_{122-489}$, NP_{26-489}, NP_{1-375} and NP_{1-440}. M, protein molecular weight marker. (a) The results obtained with polyclonal NP mouse serum; (b) The results obtained with mAbs b2, an identical pattern was observed with mAbs a2 and a2s (c) The results obtained with mAbs b4s, an identical pattern was observed with mAbs b3, (d) The results obtained with mAbs c1.

In general, antigenic sites would normally be located on the exposed surface of a protein molecule [27]. Thus, the findings of this study were partly in agreement with the observation made by Kho *et al*. [10] when one of the antigenic sites was localized within the C-terminal end of NP. Kho *et al*. [10] observed that the peptide fused to the C-terminal end of NP of NDV was located on the surface of NP structure under the electron microscopy indicating that the C-terminus of NP was exposed on the surface of NP structure. Interestingly, the present study also located two antigenic sites located within the internal region of N-terminal of the NP suggesting that some of the amino acid sequences within this region were probably exposed on the surface of NP. In contrast, Kho *et al*. [28] demonstrates that a large part of the N-terminal encompassing amino acids 1 to 375 were required for proper folding to form NP herringbone-like structure which implies

that those amino acids are probably buried inside the NP structure. It is quite difficult to explain this discrepancy unless there are probably small regions within the amino acids 1 to 375 which are exposed on the surface of NP for antibodies to bind. Further studies on the NP herringbone-like structure will probably better explain this assumption. Ultimately, this data is essential towards illustrating the NP herringbone-like structure.

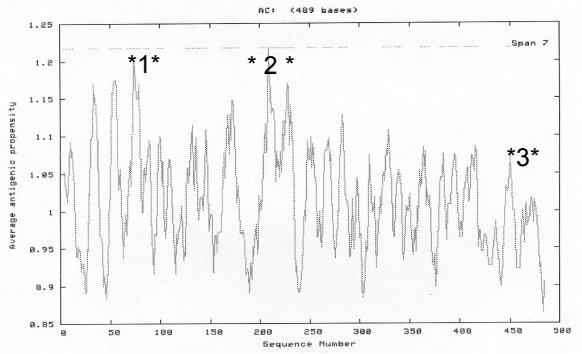

Figure 4: Antigenic peptides software analysis of NP (according to the method of Kolaskar and Tongaonkar, 1990 [23]).

There are 20 antigenic determinants predicted by the software. *1* and *2* are sequences at positions 68-81 and 199-234 amino acids (N-terminal of NP), respectively which have high antigenic propensity value. Antigenic determinants at the C-terminal (*3*; 446-452 amino acids) of NP have low antigenic propensity value.

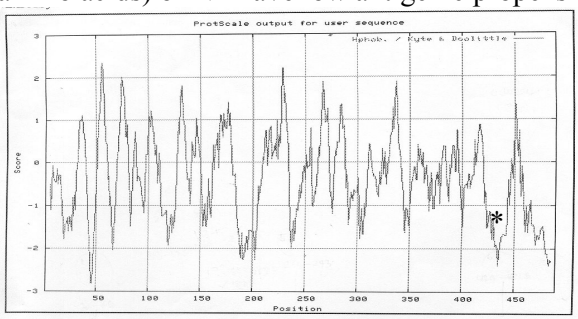

Figure 5: Hydrophobic sequence software analysis of NP (according to the method of Kyte and Doolittle, 1982 [24]).

*, Predicted antigenic site at the C-terminal position 446-452 amino acids is quite hydrophobic.

4. CONCLUSIONS

In this study, three different NP antigenic sites were localized by a panel of mAbs, one at the C-terminal of NP and two at the N-terminal of NP. To be exact, the antigenic site at the C-terminal was located within amino acids 441 to 489 of C-terminal and the other two antigenic sites at the N-terminal of NP protein were located within amino acids 26 to 121 and 122 to 375 residues.

5. ACKNOWLEDGEMENT

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6. REFERENCES

[1] Heggeness, M.H., Scheid, A., and Choppin, P.W. 1981. The relationship of conformational changes in the Sendai virus nucleocapsid to proteolytic cleavage of the NP protein. Virology 128: 105-117.

[2] Hamaguchi, M., Yoshida, T., Nichikawa, K., Naruse, H., Nagai, Y.1983. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 128: 105-117.

[3] Lamb, P.A. & Kolakofsky, D. 1996. Paramyxoviridae: The viruses and their replication. In: B.N. Fields, D.M. Knipe & P.M. Howley (Eds.) Field Virology, 3rd edn. Vol 1 (pp.1177-1203) Philadelphia: Lippincott-Raven.

[4] Marx P.A., Portner, A., and Kingsbury, D. 1974. Sendai virion transcriptase complex: polypeptide composition and inhibition by virion envelope proteins. Journal of Virology 13: 107-112.

[5] Markwell, M.A.K., and Fox, C.F. 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. Journal of Virology 33: 152-166.

[6] Kho, C.L., Tan, W.S., Yusoff, K. 2001. Sequence analysis of the nucleoprotein of a Newcastle disease virus heat resistant strain: Comparison with other members of the *Paramyxoviridae*. *J. Biochem. Mol. Biol. & Biophys*. 5: 463-471

[7] Seal, B.S., Crawford, J.M., Sellers, H.S., Locke, D.P., King, D.J. 2002. Nucleotide sequence analysis of the Newcastle disease virus nucleocapsid protein gene and phylogenetic relationships among the *Paramyxoviridae*. *Virus Res*. 83: 119-129.

[8] Pham, H.M., Chang, K.S., Mase, M., Ohashi, K., Onuma, M. 2004. Molecular characterization of the nucleocapsid protein gene of Newcastle disease virus strains in Japan and development of a restriction enzyme-based rapid identifying method. *Arch. Virol*. 149: 1559-1569.

[9] Errington, W., and Emmerson, P.T. 1997. Assembly of recombinant Newcastle disease virus nucleocapsid-like structures is inhibited by the phosphoprotein. Journal of Virology, 78: 2335-2339.

[10] Kho, C.L., Tan, W.S., Yusoff, K. 2001. Production of the nucleocapsid protein of Newcastle disease virus in Eschesichia coli and its assembly into ring- and nucleocapsid-like particles. *J. Microb*. 39: 293-299.

[11] Buchholz CJ, Spehner D, Drillien R, Neubert WJ, Homan HE. The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. J. Virol. 1993, 67, 5803-5812.

[12] Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., and Wilkinson, G.W. 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. Journal of General Virology 74: 1439-1444.

[13] Errington, W., Steward, M., and Emmerson, P.T. 1995. A diagnostic immunoassay for Newcastle disease virus based on the nucleocapsid protein expressed by a recombinant baculovirus. Journal of Virological Methods 55: 357-365.

[14] Iorio, R.M. & Bratt, M.A. 1983. Monoclonal antibodies to Newcastle disease virus: Delineation of four epitopes on the HN glycoprotein. *J. Virol*. 48: 440-450.

[15] Russell, P.H., Griffiths, P.C., Goswami, K.K.A., Alexander, D.J., Cannon, M.J., Russell, W.C. 1983. The characterization of monoclonal antibodies to Newcastle disease virus. *J. Gen. Virol.* 64: 2069-2072.

[16] Nishikawa, K., Hanada, N., Morishima, T., Yoshida, T., Hamaguchi, M., Toyoda, T., and Nagai, Y.1987. Antigenic characterization of the internal proteins of Newcastle disease virus by monoclonal antibodies. Virus Research 7: 83-92.

[17] Collins, M.S., Alexander, D.J., Brockman, S., Kemp, P.A. and Manvell, R.J. 1989. Evaluation of mouse monoclonal antibodies raised against an isolate of the variant avian paramyxovirus type 1 responsible for the current panzootic in pigeons. Archives of Virology 104: 53-61.

[18] Russell, P.H. and Alexander, D.J. 1983. Antegenic variation of Newcastle disease virus strains detected by monoclonal antibodies. Archives of Virology 75: 243-253.

[19] Alexander DJ, Manvell RJ, Lowings JP, Frost KM, Collins MS, Russell PH, Smith JE. Antigenic diversity and similarities detected in avian paramyxovirus type 1 (Newcastle disease virus) isolates using monoclonal antibodies. Avian Pathol 1997, 26, 399-418.

[20] Panshin, A., Shihmanter, E., Weisman, Y., Orvell, C., Kydyrmanov, A., Asanov, N., Daulbaeva, K., Sayatov, M., Lipkind, M. 2000. Antigenic characterization of the nucleocapsid protein of Newcastle disease virus by means of a new panel of monoclonal antibodies. *Comp. Immuno. Microb. Infec. Dis.* 23: 209-220.

[21] Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

[22] Harlow, E., and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

[23] Kolakaskar, A.S., and Tongaonkar, P.C. 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS letter 276: 172-174.

[24] Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157: 105-132.

[25] Panshin, A., Shihmanter, E., Weisman, Y., Orvell, C., Kydyrmanov, A., Asanov, N., Daulbaeva, K., Sayatov, M., and Lipkind, M. 2000. Antigenic characterization of the nucleocapsid protein of Newcastle disease virus by means of a new panel of monoclonal antibodies. Comparative Immunology, Microbiology and Infectious Diseases 23: 209-220.

[26] Gill, D.S., Takai, S., Portner, A., and Kingsbury, D.W. 1988. Mapping of antigenic domains of Sendai virus nucleocapsid protein expressed in *Escherichia coli*. Journal of Virology 62: 4805-4808.

[27] Reichlin, M. 1975. Amino acid substitution and the antigenicity of globular proteins. Advances in Immunology 20: 71-123.

[28] Kho, C.L., Tan, W.S., Tey, B.T.,Yusoff, K. 2003. Newcastle disease virus nucleocapsid protein: self-assembly and length determination domains. *J. Gen Virol.* 84:2163-2168.

[29] Mebatsion, T., Koolen, M.M., deVan, L.T.C, de Haas, Niels, Braber, M., Romer-Oberdorfer, A., den Elzen, P.V., der Marel, P.V. 2002. Newcastle disease virus (NDV) marker vaccine: an immunodominant epitope on the nucleoprotein gene of NDV can be deleted or replaced by a foreign epitope. 2002. J. Virol. 76: 10138-10146.